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13. ABSTRACT (Maximum 200 Words)

This project seeks to develop flow cytometric methods for monitoring of nuclear hormone receptor expression in human breast tumors. Data has been collected on estrogen, androgen and vitamin-D receptor expression in breast tumors from female and male patients. Gated analyses were carried out to compare receptor expression in diploid and aneuploid sub-populations. A manuscript based on these observations is now in press.

In a parallel study, we have sought correlation between expression of nuclear hormone receptors and patient characteristics of breast tumors from Indian patients. A manuscript has been submitted for publication.

We have used a recently developed NASA/ACS flow cytometers to analyze nuclei from breast tumors for electronic nuclear volume and DNA content. Our data shows that multiparametric analysis of electronic nuclear volume versus DNA content can be used to categorize subsets of ductal carcinoma in situ. A manuscript based on these observations is being submitted for publication.
In the final year of this project (no-cost extension) we will use multiparametric analysis to seek correlation between expression of nuclear parameters such as nuclear hormone receptors (e.g., PgR, AR, ER) expression, oncogene (e.g., p53) and proliferation markers (e.g., KI-67) and DNA ploidy of formalin fixed/paraffin embedded archival breast tumors.

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Table of Contents

PLEASE NOTE: THIS PROJECT HAS RECEIVED A NO-ADDITIONAL COST EXTENSION TO 30TH JUNE 2004. The following report is an annual report instead of the final report.

INTRODUCTION: Flow cytometry has become an important technique for rapid monitoring of cellular receptor expression and proliferation in hematological tumors. In contrast flow cytometry has been of limited use for the analysis of human solid tumors. Except for determination of DNA content and proliferation, monitoring of other cellular parameters by flow cytometry in breast tumors has been difficult. Most of these difficulties are posed by architectural characteristics of breast tumors where the tumor cells are embedded in stromal components containing infiltrating lymphoid cells and other non-cellular elements such as collagen. As it is comparatively easy to isolate nuclei from human breast tumors, we have worked on the hypothesis that one can use flow cytometry for monitoring the expression of nuclear markers of clinical significance. This could include nuclear hormone receptors (e.g., estrogen, progesterone, vitamin D) in combination with measurement of nuclear volume and DNA content. As the house keeping oncogenes, p53 and the proliferation marker, Ki-67 have important prognostic significance and are also expressed in nuclei, our objective was to develop multiparametric methods for rapid analysis of nuclear markers by flow cytometry.

Work accomplished to date has focused on 1. Monitoring of nuclear hormone receptor expression in breast tumors by flow cytometry and 2. Rapid monitoring of nuclear volume and DNA content of fresh and formalin fixed archival breast ductal carcinoma in situ. We believe development of this method may refine histopathological grading of ductal carcinoma in situ.

The flow cytometric methods we have developed are rapid, highly sensitive and can determine not only the percentage of receptor positive nuclei in a heterogeneous breast tumor population but also measure antigen density of the individual nuclei and subpopulations. By multiparametric analysis, we have correlated expression of different markers with DNA aneuploidy. We believe flow cytometric analysis of nuclear hormone receptors and nuclear volume in combination with rapid and high-resolution analysis of DNA content can be an important quantitative multiparametric method for diagnostic and prognostic studies in breast cancer.

BODY:

Our major specific aims were to: 1. Collect breast tumor biopsies and archival tissue blocks from local and outside repositories. 2. Refine preparatory methods for flow cytometric analysis of nuclei obtained by enzymatic digestion of archival breast tumors 3. Study the resulting single nuclei for marker expression (estrogen, androgen and vitamin-D hormone receptors, Ki-67, p53) and DNA content (aneuploidy and S-phase fraction).

Breast Tumor Collection was based on samples collected from the Jackson Memorial Medical Center, Miami, Suburban Hospital, Bethesda, MD, and NIH sponsored CHTN tissue network, Birmingham, AL. Most of the samples from our local patients represent a broad spectrum of racial and ethnic populations unique to South Florida. From these resources, frozen and formalin fixed/paraffin embedded blocks of breast tumors representing a variety of histopathological grades and types of tumors were collected. In addition we collected formalin fixed/paraffin embedded female breast tumors from India and Egypt. Male breast tumors were collected from Jamaica, West Indies. Our interest in breast tumors from India, Egypt and Jamaica was due to the reported early onset and aggressive behavior of breast tumors in these special populations.

Nuclear Estrogen Receptor Expression and DNA ploidy: This study was based on examination of more than 100 breast tumor samples of different histological grades and types collected from USA and Egypt. Thick (50 micron) and thin (5 micron) sections were cut from paraffin blocks of formalin fixed breast tumors for analysis by laser flow cytometry and immunohistochemistry respectively. Thick sections were enzymatically digested for release of nuclei, processed for antigen retrieval and studied for receptor expression and DNA content (aneuploidy and cell cycle distribution) by flow cytometry. Thin sections cut were stained by immunohistochemistry and studied for ER expression and histopathological grading.

Data from immunohistochemistry and flow cytometry was analyzed for percentage of ER positive cells and DNA content of the subpopulations. Flow cytometric data was compared with immunohistochemical determinations on ER positive or negative status of the tumors examined under a microscope. Correlation between ER expression by flow cytometry and the immunopathological evaluation of the tumors was undertaken. Dr. Khayat, of the National Cancer Institute in Cairo was the primary investigator on this project. He is preparing a manuscript based on observations in this study for possible publication.

Androgen, Vitamin-D Receptor Expression and DNA content of Human Breast Tumors: In the second year of the project, Dr. Rao a UICC/ICRETT International scholar and Dr. Poonam Arya, Research Associate used our flow cytometric methods to monitor expression of androgen and vitamin-D nuclear hormone receptors in breast tumors. Dr. Rao focused on breast tumors collected in Manipal, India while Dr. Arya worked mostly on breast tumor samples from USA and Jamaica. We had chosen the Indian and the Jamaican breast tumors for study due to reported early onset and aggressive course of breast tumors in these populations. Methods for both antigen retrieval and staining had to be modified for the analysis of tumors obtained from outside USA as the fixation and embedding methods used in these labs differed from those used in our labs.

Thick sections were enzymatically digested for isolation of nuclei, which were then processed for antigen retrieval and stained with the anti-AR or vitamin-D primary antibodies and labeled secondary antibodies. Thin sections were stained for immunohistochemistry and studied for AR/VDR expression and histopathological grading. Gated analysis was used to compare the expression of these receptors in sub-populations with diploid and aneuploid DNA content.

Out of the 25 **female breast tumors** analyzed, 13 tumors had diploid DNA content, while 12 had distinct aneuploid sub-populations with triploid or hypo-tetraploid DNA content. The percentage of AR positive nuclei decreased with increase in tumor grade. Aneuploid subpopulations had higher percentage of AR positive nuclei as compared to diploid sub-populations. The mean percentage of AR positive nuclei in diploid, triploid and in tumor sub-populations with greater than 4N DNA content was 55±19, 63±17 and 85±18 respectively. In multiploid tumors, aneuploid nuclei had higher AR expression than diploid nuclei.

Besides determining the percent of AR positive nuclei in a sub-population, we compared the ratio of the mean log fluorescence channel (MFC) value of the isotype and the antibody treated samples as a measure of AR density. Variable MFC ratios were obtained. For example, in diploid tumors the MFC ratio varied from 1.87 to 6.5. In tumors with triploid sub-populations and the near-tetraploid tumors the MFC ratio ranged between 2.28 to 6.80 and 2.3 to 6.79, respectively. Data on AR expression of some selected female breast tumors is shown in figure 1 (appendix I).

Out of 33 **male breast tumors** analyzed, 25 tumors were predominantly diploid and the remaining 8 tumors contained distinct aneuploid populations. In diploid tumors, the percent of AR positive nuclei varied from 5 to 61 with a mean of 31±14. In triploid sub-populations, the range was from 23 to 64 with a mean of 44±15 and in subpopulations with greater than 4N DNA content; the range was from 16 to 61 with a mean of 40±16. AR expression in male breast tumors was in general lower than that of the female breast tumors. Figure 2 (appendix 2) shows AR expression in male breast tumors.

The percentage of **vitamin D receptor** positive nuclei varied from 28 to 86 with a mean of 66±21 in diploid nuclei. In triploid tumors, the range was 68 to 91 with a mean of 70±16. In sub-populations with greater than 4N DNA content the range was from 36 to 91 with mean of 63±20. VDR expression in breast tumors was highly variable and diploid sub-populations had higher VDR expression than aneuploid sub-populations. Figure 3 (appendix 3) shows data on VDR expression in female breast tumors. A revised manuscript has been sent for publication in Clinical Cytometry (4).

Dr. Rao, UICC International technology transfer fellow in our lab. used the flow cytometric methods to compare and correlate the expression of AR and vitamin-D in paraffin embedded breast tumors from Indian Patients with other clinical parameters. Paraffin embedded blocks from 76 primary infiltrating ductal (ID) breast carcinoma and benign breast tumors from the Department of Pathology, Kasturba Medical College, India were used for this study. Approximately 44% of the ID tumors were diploid while the remaining tumors had a sub-population of nuclei with distinct triploid (36%) or tetraploid (10%) DNA content. Percent AR positive nuclei ranged from 16-66% in the ID tumors and 36-67% in benign tumors. The percent of vitamin-D receptor positive nuclei was 14-89% and 2-75% in ID tumors and benign tumors, respectively. Benign tumors had significantly higher AR and significantly lower Vitamin-D expression than nuclei from the malignant tumors. There was a strong correlation between the percent of receptor positive nuclei and the antigen density as measured by the mean log fluorescence in benign and the intraductal tumors.

The pooled data from patients of pre-and post-menopausal group with an average age of 47 ± 13.2yrs (28 to 80 years) revealed percentage of vitamin-D positive nuclei decreased with the patients' age. However, a similar co-relation between patients' age and AR expression was not seen. Interestingly, both AR and vitamin-D expression decreased with an increase in patients age in the pre-menopausal group, and AR expression increased with the increase in patients age in post-menopausal group. The AR-vitamin-D expression and tumor grade in the pooled data revealed weak correlation. In the pre-menopausal women, a weak correlation between vitamin-D expression and clinical stage was observed. The percentage of vitamin-D positive nuclei decreased and AR positive nuclei increased along with the tumor grade. Whereas, both the percent AR/ vitamin-D positive nuclei decreased among the women in post-menopausal group with advanced stage of malignancy. No correlation was seen between DNA ploidy and receptor expression. The percentage of AR/ vitamin-D positive nuclei decreased in both pre and post-menopausal group with the increase in tumor size. No statistically significant correlation was observed between other clinical parameters such as tumor grade, nodal status, and metastasis and AR/Vitamin-D expression. A manuscript based on this data has been submitted for publication (5).

Nuclear Volume versus DNA content of nuclei isolated from Human breast tumors.

We have described development and use of a high-resolution flow cytometer, which can simultaneously measure nuclear volume and DNA content of tumor nuclei in suspension (1-3). This instrument by using multiparametric analysis of electronic nuclear volume versus DNA content can differentiate between normal and tumor cells and identify tumor cells in secondary sites such as lymph nodes or bone marrow of breast tumor patients. Our first effort was to develop staining methods for optimizing multiparametric analysis of breast tumor cells. We explored different combinations of DNA binding dye concentrations, pH and tonicity to identify conditions, which provide the best possible data for multiparametric and simultaneous evaluation of nuclear volume and DNA content of nuclei prepared from a tumor biopsy (2).

For histopathological grading of human breast ductal carcinoma in situ, nuclear size, chromatin texture and necrosis are considered to be three important parameters (6,7). At present most of the histopathological studies are carried out visually using a microscope and thus are slow and often based on examination of a small number of cells.

In the NASA/ACS flow cytometer, simultaneous measurement of electronic volume versus DNA content can identify sub-populations in a heterogeneous solid tumor and possibly discriminate between normal and tumor cells at metastatic sites (3). We have recently developed methods for rapid determination of nuclear size and DNA content of nuclei isolated from cryopreserved and formalin fixed-paraffin embedded human breast tumors. Our preliminary data showed that this method could be used to detect differences in nuclear volume and DNA content of tumor cells with a level of precision unattainable with standard flow cytometric analysis in conventional flow cytometers. Our working hypothesis is that measurement of nuclear volume versus DNA content of human ductal carcinoma in situ in combination with monitoring the expression of nuclear hormone receptors by our flow cytometric methods can be a useful tool for accurate grading of breast tumors.

We have completed a study of nuclear volume in ductal carcinoma in situ and the following figure 4 shows a representative set from a manuscript in preparation. DNA histograms (Figure 4, A-D) and contour plots of DNA vs. Electronic nuclear volume (Figure 4,E-H) are of nuclei isolated from normal breast tissue (A, E), and from tumor biopsies of patients diagnosed with ductal carcinoma in situ (DCIS, B-D, F-H). Trout red blood cells were added to the samples as an internal standard and recorded at channel # 50 (Figure 4, A-C) or at # 29 (Figure 4, D).

In normal breast tissues, nuclei with diploid (2C) DNA content were recorded at channel # 82 with a corresponding small peak of cells with G_2/M DNA content at channel # 165. In contour plots, the normal diploid breast nuclei had electronic volume similar to that of the TRBC used as controls (Figure 4E).

Electronic Nuclear Volume vs. DNA content contour plots of the three representative DCIS samples shown in Figure 4 (F-H) show the variation in electronic volume of the nuclei with diploid, tetraploid and hexaploid DNA content. In contour plot 4F, the nuclei with both diploid and tetraploid DNA content were much larger than TRBC but had similar electronic volume. In contrast in contour plot 4G, while most of the nuclei with diploid DNA content had electronic volume similar to that of the TRBC, the nuclei with tetraploid DNA content were 2 to 3 times larger.

DNA histogram in figure 4D shows TRBC at channel 29, a diploid peak at channel 52 and an aneuploid (hexaploid) peak at channel 150. Contour plot 4H shows that in contrast to the nuclear volume of the tetraploid nuclei in figure 4G, the hexaploid nuclei in this tumor had relatively smaller volume.

Identification of Sub-populations Based on Nuclear Volume vs. DNA Content:

Plotting of electronic nuclear volume vs. DNA content in contour plots can reveal the presence of sub-populations which may not be readily recognizable in DNA histograms or in light scatter vs. DNA plots. As shown in DNA histogram 5A, a distinct hyper-diploid population with DNA index of 1.15 is recognizable as nuclei with large electronic volume in the nuclear volume vs. DNA contour plots shown in figure 5B. In DNA histogram 5C, the twin peaks of nuclei with tetraploid DNA content (DI, 1.79, 1.98) are resolved as two distinct populations with similar electronic nuclear volume in the contour plot 5D.

In DNA histogram 5E, three main peaks of diploid, hypo-tetraploid (DI 1.73) and tetraploid (DI 2.03) DNA content are seen. The ENV vs. DNA plot of this tumor in Fig 5F show that both the diploid and the hypo-tetraploid populations had sub-populations that differ in their nuclear volume. On the basis of electronic nuclear volume vs. DNA contour plots one can recognize at least eight sub-populations in this DCIS sample.

We have submitted a grant application to NIH (June 01,2003 deadline) for continuation of studies on grading of DCIS on the basis of nuclear volume and DNA content. A manuscript based on analysis of nuclear volume versus DNA content is being prepared for submission for publication (8).

Correlation of nuclear marker expression: In the final phase of the project, (June 2003-May 31, 2004), we will focus on multiparametric flow analysis for correlating expression of the nuclear markers with DNA content and other markers such as those of proliferation (Ki-67) and oncogen p53. As pointed out in the previous review of our progress report, we had not addressed this part of the project as it became evident that use of nuclear volume as a parameter would add a new dimension to our studies. However, we had to wait for some modifications and upgrading of the instrument so it can provide two-color analysis in combination with measurement of nuclear volume.

As shown in Figures 4 and 5, the use of nuclear volume as a parameter is far more superior than monitoring of nuclear size by side scatter. With the routine availability of nuclear volume as a parameter, it was decided that these studies should be carried out on the NASA/ACS flow cytometer, as nuclear volume was probably a more valuable marker in tumor grading. The initial prototype of this instrument had a single channel solid-state detector for monitoring of fluorescence of nuclei stained with DAPI. This detection system although good enough for detection of DNA fluorescence, was not adequate for monitoring fluorescence of markers such as p53 and Ki-67. In year 2002, the NASA/ACS instrument was modified by the addition of two highly sensitive photo multiplier tubes for detection of low-level two-color fluorescence. The hydraulics was improved by addition of an automatic sample introduction system and other refinements in hardware and software has made this a valuable instrument for the proposed multiparametric studies. After testing of these additional refinements, we ran experiments to see if labeled antibody fluorescence can be used in multiparametric setting of this instrument with nuclear volume and DNA content. Nuclei isolated from paraffin embedded breast tumors were stained with phycoerythrin labeled anti-androgen and progesterone antibodies. Our preliminary data shows that we can now simultaneously measure nuclear volume and fluorescence of a labeled antibody in this instrument. We have started this final part of the project and propose to finish these studies in the present year. For this purpose we have asked for no additional cost extension from April 1 2003 to May 31, 2004. For the proposed study we have formalin fixed paraffin embedded breast tumor, which are being cut and used for isolation of nuclei. We have also requested CHTN for fresh (frozen) samples of DCIS for the proposed correlative studies.

KEY RESEARCH ACCOMPLISHMENTS:

- Analyzed 100 breast tumors of different grades and types for the expression of nuclear estrogen receptor expression. Flow cytometric data is being compared and correlated with immunohistochemical data.
- Studied androgen and vitamin-D expression in female and male breast tumors collected from South Florida, India, Egypt and Jamaica. A manuscript has been sent for publication to Clinical Cytometry and the reviewers request for some additional information is being addressed for a revised submission.
- Studied nuclear androgen and vitamin-D expression in Indian breast tumors and correlated expression with clinical parameters of age, menopause, tumor grade, tumor type and co-expression of AR and VDR receptors. A manuscript based on these observations has been submitted to Breast Journal for publication.
- Developed methods for simultaneous analysis of nuclear volume and DNA content of ductal carcinoma in situ by a high resolution flow cytometer. Completed study of more than 100 ductal carcinoma in situ for correlation of nuclear volume vs. DNA

content with histopathological grading of the tumors. Data is being analyzed for possible publication.

- Developed staining protocols for simultaneous staining of breast tumor nuclei with antibodies for nuclear receptors and correlation of receptor expression with nuclear volume. This part of the stated aim was delayed due to upgrading of instrumentation for performance of two color analysis.

REPORTABLE OUTCOMES:

- Flow cytometric protocols have been developed for monitoring of nuclear hormone receptor expression in archival formalin fixed/paraffin embedded breast tumors.
- Androgen expression is reduced in high-grade tumors as compared to low-grade tumors.
- In multiploid female and male breast tumors, aneuploid sub-populations have higher percent of AR positive nuclei than diploid populations.
- Male breast tumors have relatively lower AR expression than corresponding female breast tumors.
- In female breast tumors, vitamin D receptor expression was higher in diploid than in aneuploid nuclear sub-populations.
- In multiploid female breast tumors, the aneuploid sub-populations did not have significantly greater percentage of vitamin-D positive nuclei than the diploid sub-populations in the same tumor.
- Nuclear volume versus DNA content may be a useful parameter to identify sub-sets of human breast ductal carcinoma in situ. Correlation of nuclear volume with tumor grade may allow us to refine the grading of DCIS.

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APPENDICES:

I. Figure 1. Androgen Receptor Expression in Female Breast Tumors.
II. Figure 2. Androgen Receptor Expression in Male Breast Tumors.
III. Figure 3. Vitamin D Receptor Expression in Female Breast Tumors.
IV. Figure 4. Electronic Nuclear Volume vs. DNA in DCIS
V. Figure 5. Electronic Nuclear Volume vs. DNA in subpopulations
VI. Mss. Androgen and Vitamin-D receptor expression in archival human breast tumors. Krishan et al. Revised mss submitted to Clinical Cytometry

AR EXPRESSION IN FEMALE BREAST TUMORS

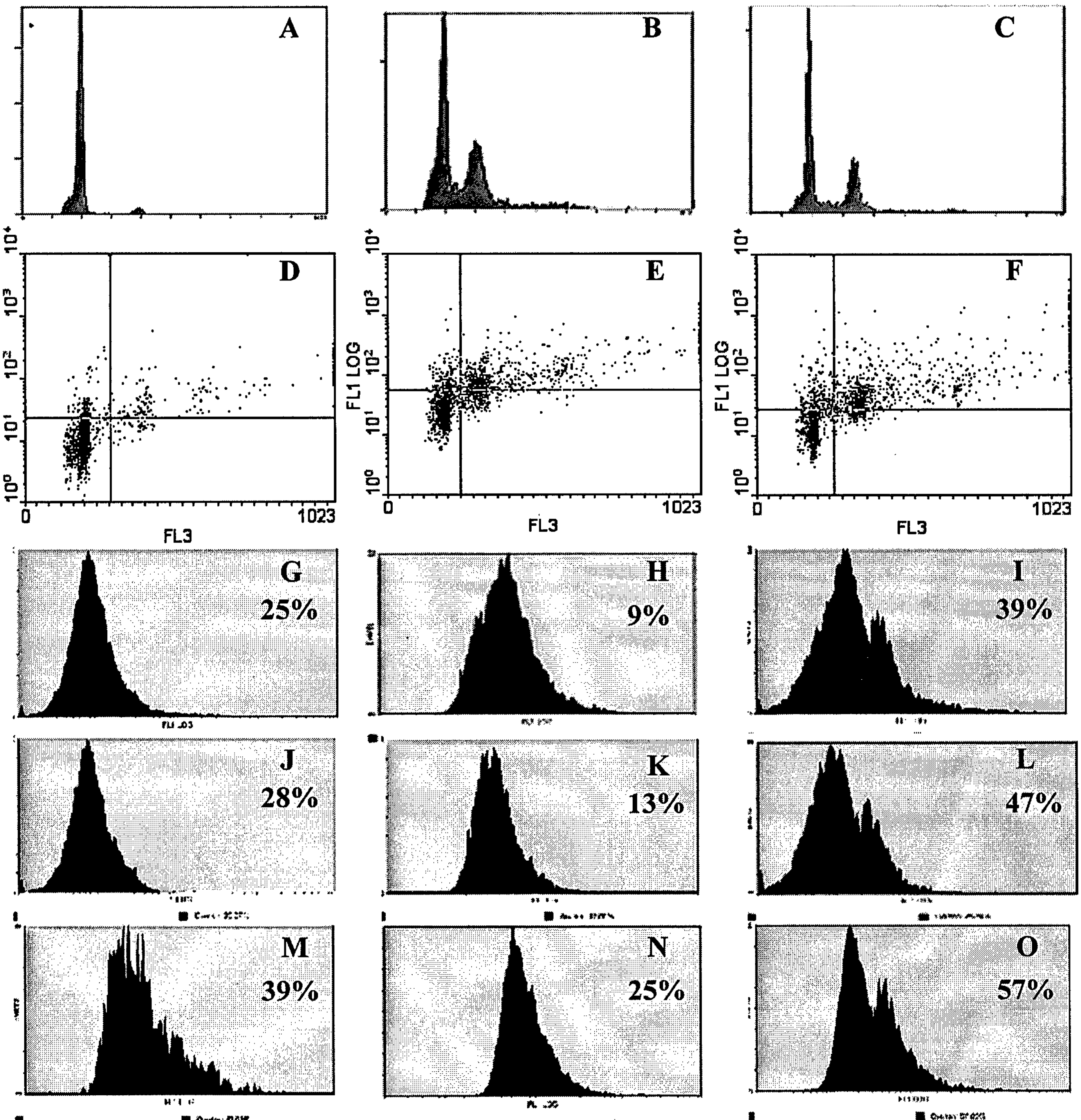

Figure 1

Androgen Receptor Expression in Female Breast Tumors.
Figures A-C are DNA histograms of diploid and aneuploid tumors. Figures D-F are scatter plots of AR expression (Y-axis) vs. DNA content (X-axis). The horizontal line is the gate used to subtract 95 percent of the fluorescent cells from the isotype controls. Histograms G-O are from analysis of the isotype and the antibody treated nuclei by the Overton's method. G-I are of the total population, while J-L and M-O are respectively, of the diploid and aneuploid sub-populations.

APPENDIX-I

APPENDIX-II

AR EXPRESSION IN MALE BREAST TUMORS

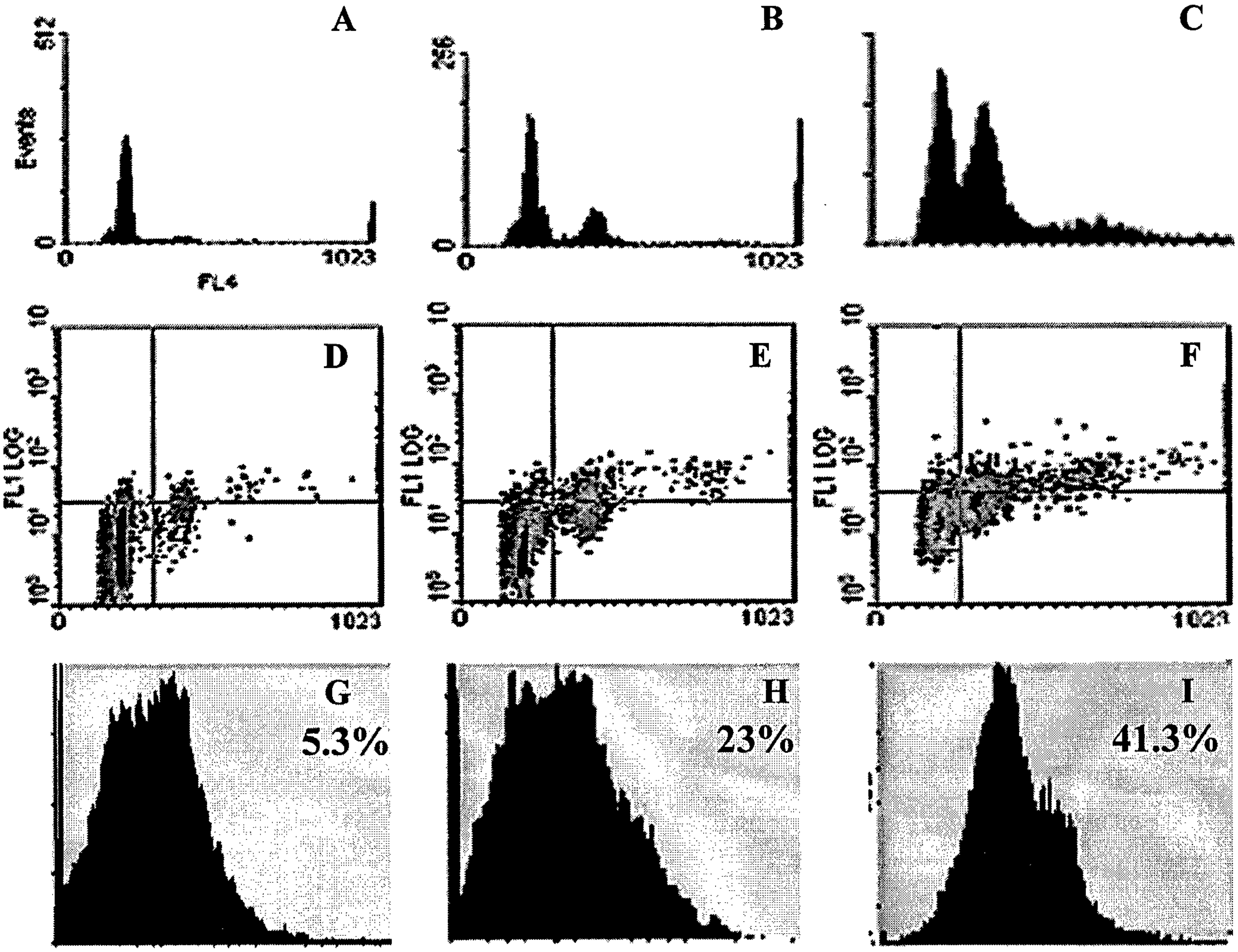

Figure 2

Androgen Receptor Expression In Male Breast Tumors.
Histograms (A-C) show representative DNA histograms of breast tumors from male patients with diploid and aneuploid subpopulations. The scatter plots (D-F) show DNA content (X-axis) vs. androgen receptor expression (Y axis) of nuclei incubated with the anti-androgen antibody. Dots above the horizontal line indicate nuclei with positive receptor expression. G-I are Overton's analysis of the AR expression in isotype and antibody treated samples.

APPENDIX-III

VDR EXPRESSION IN FEMALE BREAST TUMORS

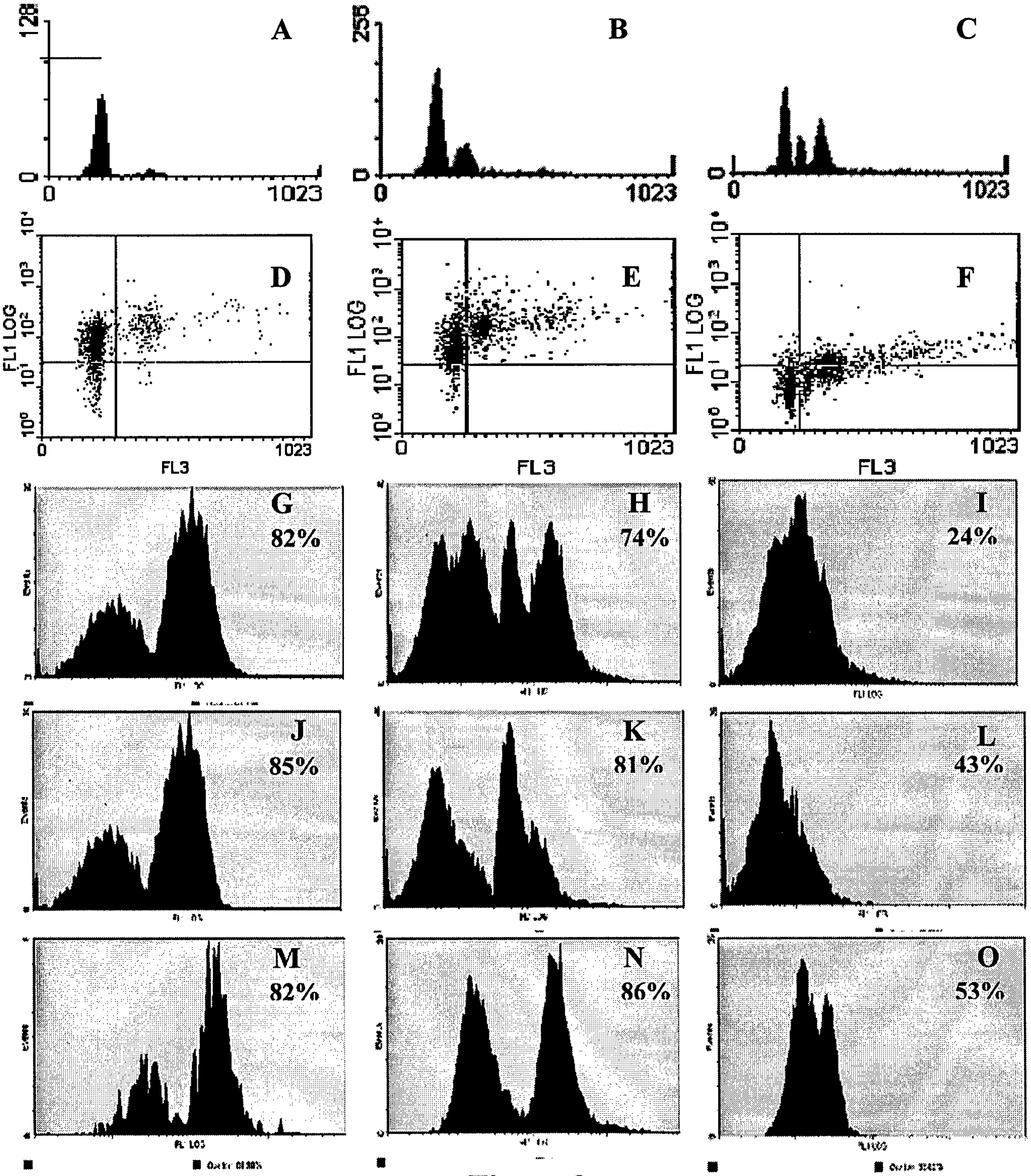

Figure 3

Vitamin D receptor expression in Breast Tumors

DNA histograms (A-C) show breast tumor with diploid, triploid and multiploid sub-populations. D-F are scatter plots of VDR vs. DNA content. Histograms G-I are Overton's analysis of the total population, J-L of diploid sub-populations and M-O of the aneuploid populations.

APPENDIX-IV

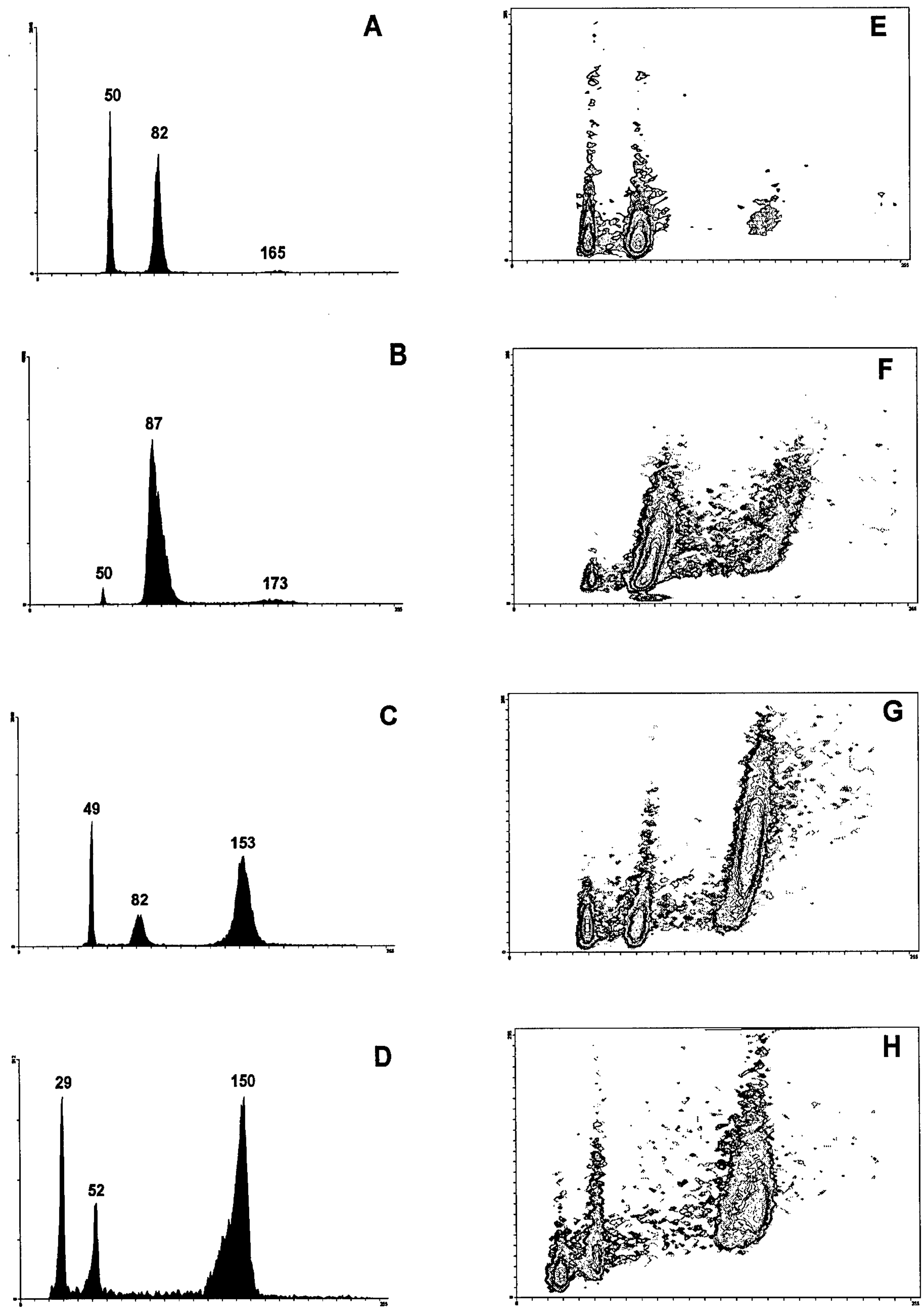

Figure 4

APPENDIX-V

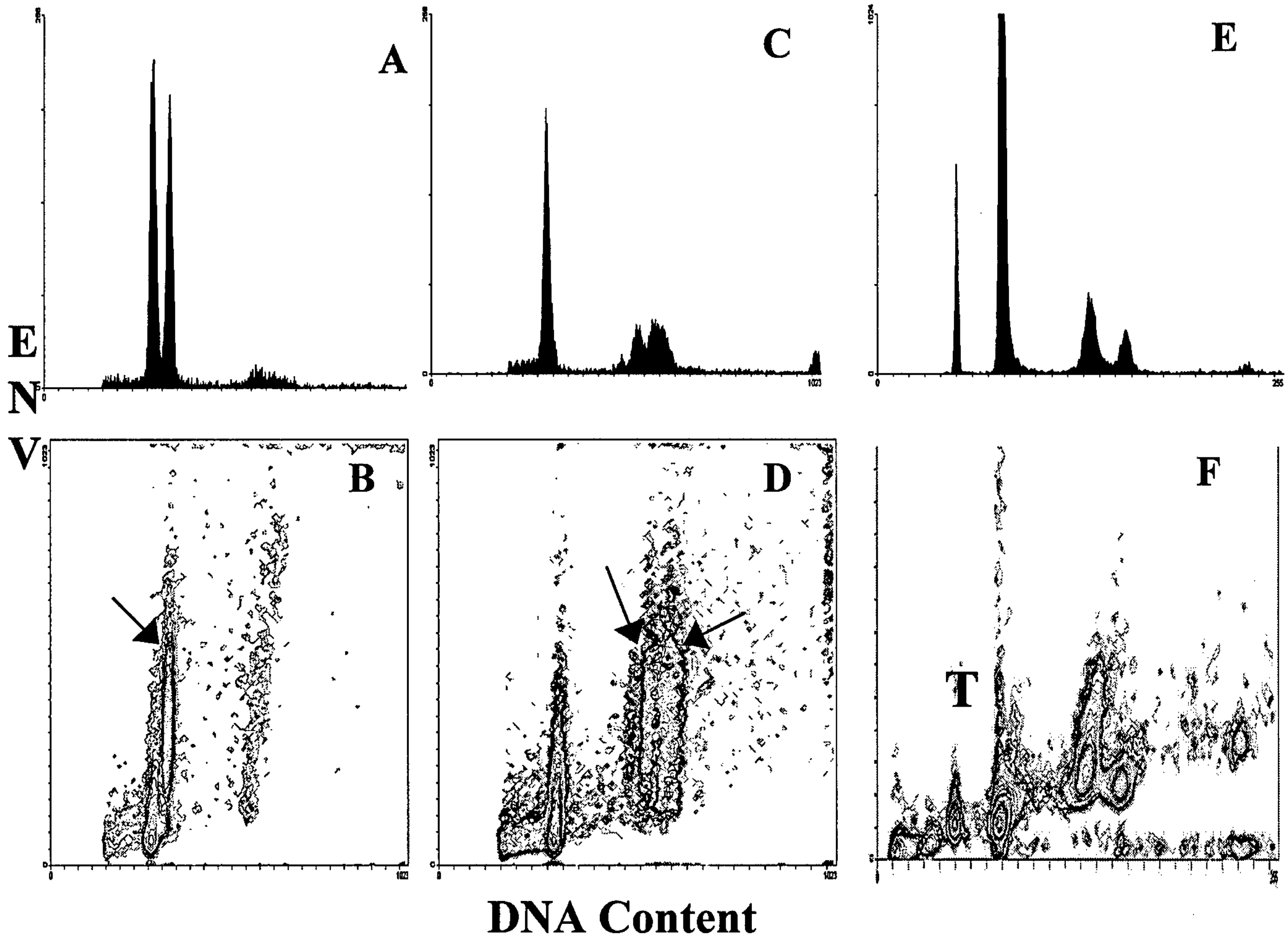

Figure 5

APPENDIX-VI

Androgen and Vitamin D Receptor Expression in Archival Human Breast Tumors

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ABSTRACT

Purpose: Hormone receptor expression in human breast tumors is of diagnostic and prognostic significance. Flow cytometric methods have been recently developed for quantitative analysis of receptor expression in formalin-fixed paraffin-embedded archival tissues. The present study was undertaken to use these methods for quantitation of androgen and vitamin D receptor expression in human male and female breast tumors. Gated analysis was used to compare the expression of these receptors in sub-populations with diploid and aneuploid DNA content.

Experimental Design: Nuclei isolated from thick sections of formalin-fixed, paraffin-embedded human male and female tumors by pepsin digestion were treated for antigen unmasking and incubated with antibodies to androgen and vitamin-D receptors. Propidium iodide was used for staining of nuclear DNA. Two-parameter flow cytometric analysis was used to determine the percent of receptor positive nuclei with fluorescence greater than 95 % of the isotype nuclei. Mean log fluorescence channel values were used for comparing antigen density of the isotype and the antibody treated nuclei. Gated analysis based on DNA content of the diploid and the aneuploid sub-populations was used for comparing receptor expression in sub-populations.

Results: Low-grade female breast tumors had a higher percentage of AR positive nuclei than high-grade tumors. In multiplied tumors, aneuploid nuclei had higher AR expression than diploid nuclei. In male breast tumors, AR expression was in general lower than that of the female breast tumors. VDR expression in breast tumors was highly variable and diploid sub-populations had higher VDR expression than aneuploid sub-populations.

Breast cancer is one of the leading malignancies and as breast tissue in general is hormone sensitive, hormonal therapy is used for both prevention and treatment of this malignancy (1). Receptors for estrogen (ER), progesterone (PR), androgen (AR) and vitamin D (VDR) are present in normal human breast and tumor cells (2-7). The predictive and prognostic significance of ER and PR expression in breast cancer has been elucidated in several recent studies (7, 8). In comparison, significance of AR and VDR expression in human breast tumors is less clear. Several authors have used cytosolic (dextran-charcoal), RT-PCR or immunohistochemical assays (IHC) to study androgen receptor (AR) expression in human breast tumors. Kuenen-Boumeester et al. (4) reported that 76% of breast tumors are AR positive while 9% of AR positive tumors are negative for ER and PR expression. In 13 % of tumors (all grade III), no detectable expression of ER, PR or AR was seen. In a recent IHC study, Selim et al. (8) reported that 19 out of 57 ductal carcinoma in situ (DCIS) were AR positive and no association between AR and ER expression or AR expression and degree of differentiation was seen.

Although the role of VDR in growth regulation and differentiation of breast tumor cell lines has been confirmed in several in vitro studies (9), conflicting reports have appeared on the prognostic value of VDR expression in human breast tumors (10 –12). Freake et al. (6) reported that in human breast tumor cells, VDR expression was at concentrations higher than previously described, and Buras et al. (13) reported that high VDR expression was seen in well-differentiated tumors as compared to less differentiated tumors.

Most of the earlier work on hormone receptor expression was based on ligand binding assays, which measure cytosolic receptor expression. With the availability of receptor specific antibodies, immunohistochemistry has replaced ligand-binding assays for clinical evaluation of hormone receptor expression in human breast tumors (14, 15). As ligand-binding assays are based on determination of the receptor content in the cytosol, they cannot differentiate between receptor expression of the normal and the tumor cells. The IHC methods are based on examination of a small number of cells under a microscope and are at best semi-quantitative.

Flow cytometry is extensively used for monitoring of receptor expression in human hematopoietic cells. Flow cytometry offers the advantage that quantitative data collected by rapid analysis of a large number of cells can be used for multiparametric correlative studies. However, use of flow cytometry for monitoring of hormone receptor expression in human solid tumors has been hampered by difficulty in obtaining single cell suspensions from solid tumors. As most of the antibodies used for immunohistochemical detection of the hormone receptors are specific for the nuclear receptors, we have used enzymatic procedures for isolation of nuclei from archival paraffin embedded tumors for flow cytometric determination of hormone receptor expression. In earlier publications, we have described methods for analyses of estrogen, progesterone and androgen receptor expression (15 - 17) in human breast and prostate tumors. In the present study, we have used these flow cytometric methods for monitoring AR and VDR expression in formalin-fixed paraffin-embedded archival human male and female breast cancers.

MATERIAL AND METHODS

Formalin-fixed, paraffin-embedded human breast tumor blocks (from 25 female and 34 male patients) were obtained from the Departments of Pathology at the University of Miami and the University of West Indies in Jamaica. Data was obtained on 23 out of 25 female breast tumors and 33 out of 35 male breast tumors processed for antigen retrieval and receptor expression. Table 1 and 2 list the histopathological and immunocytochemical characteristics of the tumors analyzed. A hematoxylin and eosin stained section was used for histopathological examination and selection of material for flow cytometric studies. Two 25 μM sections from each block were deparaffinized, rehydrated in a descending ethanol series and resuspended in 2 ml of phosphate buffered saline (PBS).

Enzyme Digestion and Antigen Retrieval

For antigen unmasking (antigen retrieval), sections were incubated in citrate buffer (0.01M at pH 6.0) at 80 °C for 2 h. After cooling at room temperature for 15 min., the tissue sections were washed with PBS. For nuclear isolation, sections were digested in 3 mL of pepsin (0.05% in normal saline, pH 1.65, Cat # P-7012, Sigma, St Louis, MO) for 30 min. at 40 °C in a water bath. Tubes were periodically vortexed during the incubation. Examination of the digests under a microscope was used to confirm the isolation of nuclei. Proteolytic action was stopped by the addition of an equal amount of PBS containing 3% fetal bovine serum. The nuclear pellet retrieved by centrifugation for 16 min at 1000X g in a Beckman TJ-6 centrifuge was washed with PBS and resuspended in PBS.

Receptor Staining

250 μL of the pepsin-digested sample were incubated with 200μL of the anti-androgen antibody (MU256-UC, clone F39.4.1, BioGenex, San Ramon, CA) diluted 1:45 in PBS for 18 h at 37 °C. Mouse IgG1 Kappa (Cat. # M 5284, MOPC 21, Sigma, St Louis, MO.) diluted 1:180 in PBS, was used as the isotype control. All samples were washed with 3% fetal bovine serum in 0.05% Triton-X in PBS, centrifuged at 1000X g in Beckman TJ-6 centrifuge for 16 min. The secondary goat anti-mouse antibody (FITC conjugated, cat. # F-4143, Sigma), at a dilution of 1:80, was mixed with the nuclear suspension and the tubes were incubated in dark for 45 min. After a wash with PBS containing 0.05% Triton–X in 3% fetal bovine serum, the tubes were centrifuged and the pellets were stained with propidium iodide (50 μg/ml PI in PBS containing 1 mg/ml of RNase) at 4 °C for 20 min.

250μL of the nuclear suspension was incubated with anti-vitamin D receptor antibody (MA1-710, rat monoclonal, IgG2b, Affinity Bioreagents, Golden, CO) at 1:150 dilutions for 18 h at 4 °C. Positive control used was rat IgG (Cat. # I-8015, Sigma) at a protein concentration similar to that of the antibody. Following incubation, the pellet was washed with PBS and stained with 110μL of FITC conjugated goat anti-rat IgG (Cat. # F-6258, Sigma) at 1:80 dilutions in PBS for 35 min. in dark. Samples were counter stained with the propidium iodide solution as described above.

Flow Cytometry

Samples were analyzed in a Coulter XL flow cytometer with the standard argon ion laser excitation and filter configuration for FITC/propidium iodide dye combination. A minimum of 10,000 cells was used to generate a list mode data file. The percentage of receptor positive cells in the total and gated sub-populations were determined by the Overton's method in the EXPO–32 software obtained from Beckman Coulter Inc. As described by Overton (18), the cumulative subtraction method subtracts cells in each channel of the isotype control from that of the corresponding channels in the test histogram. The negative differences are not replaced with a zero value but negative values are added to positive differences in lower channels. The negative differences of higher fluorescence intensity and the positive differences of the lower fluorescence intensity cancel each other out. When cumulative subtraction is completed for all channels, a positive difference histogram is generated by assigning a value of zero to any channel with a residual negative difference. The percent positive is then calculated from the sum of the differences for all channels.

Immunohistochemistry

Formalin-fixed, paraffin-embedded 3 μM sections were used for immunostaining. The sections were hydrated in decreasing ethanol solutions. Endogenous peroxidase was blocked with 6% hydrogen peroxidase for 3 minutes .The slides were rinsed in water, placed in a dish containing a target retrieval solution (DacoCytomation) that was heated previously to 90°C, then placed in a steamer for 20 minutes to achieve optimal antigen retrieval. After a 30-minute cooling period, the slides were incubated with the avidin solution and, subsequently, with a biotin solution using the Biotin Blocking System (DakoCytomation) prior to application of the primary antibody. ER monoclonal antibody (DakoCytomation, M 7047) at dilution of 1:25 and AR monoclonal antibody (Biogenex, MU256-UC) at dilution of 1:20 were used for 30 minutes (Steamer) .The slides were then rinsed in buffer and incubated for 25 minutes with the linking solution (DakoCytomation LSAB+ Kit; biotinylated anti-mouse, anti-rabbit, and anti-goat). This was followed by a rinse in buffer and incubation with streptavidin peroxidase for 25 minutes. After rinsing in buffer, the slides were submerged in 3,3'-diaminobenzidine tetrahydrochloride for 5 minutes .One percent cupric sulfate was applied for 5 minutes, and the slides were counterstained with 0.1% Fast Green .The slides were then dehydrated through gradient alcohols, cleared in xylene, and coverslipped. Sections of normal female breast and normal testis were used as the positive antibody control. The results of the immunostaining were based on the nuclear staining of tumor cells. Staining was considered positive when more than 5% of tumor cell nuclei reacted with any intensity.

RESULTS

AR Expression in Female Breast Tumors

Table 1 lists the histopathology type, DNA index (DI), ER expression and staining intensity (as determined by IHC) and percent of AR positive nuclei (as determined by flow analysis) of the 23/25 tumor samples analyzed. In DNA histograms, 13/25 tumors had diploid DNA content (DI=1.0), while 12/25 contained (besides the diploid populations) distinct aneuploid sub-populations with DNA index of 1.6 (near-triploid) to 1.9 (near-tetraploid).

Figure 1A correlates tumor grade with the percent of AR positive nuclei seen in the 25 tumor samples analyzed. The lower grade tumors appeared to have a higher percentage of AR positive nuclei than tumors of the higher grade. Twenty of the ER positive tumors had AR positive nuclei ranging from a low of 9% to as high as 79%. The three ER negative samples had 35-42% AR positive nuclei.

Figure 1B plots data from gated analysis of diploid and aneuploid sub-populations of the 25 tumors from female patients. The percentage of AR positive nuclei in aneuploid subpopulations was in general higher than that of the diploid sub-populations. The mean percentage of AR positive nuclei in diploid tumors was 55 ± 19. In triploid sub-populations, the range was from 33 to 96 with mean of 63 ± 17. In tumor sub-populations with greater than 4N DNA content, the range was from 51 to 98 with a mean of 85 ± 18.

Figure 2 (top row, A-C) shows representative DNA histograms of tumors with diploid (A), triploid (B, DI=1.6) and hypo-tetraploid (C, DI=1.9) sub-populations. The scatter plots (D-F) show DNA content (X-axis) vs. AR expression (Y axis) of nuclei incubated with the anti-androgen antibody. The horizontal line in each scatter plot indicates the electronic gate used to exclude 95% of the fluorescent cells in the isotype controls. The vertical line demarcates cells with diploid and aneuploid DNA content. Dots above the horizontal line (95% percentile) indicate AR positive nuclei with receptor expression greater than that of the isotype controls.

The histograms in the lower three rows show analysis of the isotype and the AR antibody treated samples by the Overton's method. Histograms in the first row are of the total nuclear population while those in the middle and bottom are of gated sub-populations with diploid and aneuploid DNA content, respectively. In general Overton's analysis showed that the aneuploid sub-populations had higher percentage of AR positive nuclei than the diploid sub-populations.

Besides determining the percent of AR positive nuclei in a sub-population, we compared the ratio of the mean log fluorescence channel (MFC) value of the isotype and the antibody treated samples as a measure of AR density. In diploid tumors MFC ratio varied from 1.87 to 6.5. In tumors with near-triploid sub-populations, the MFC ratio was 2.28 to 6.80 while in the near-tetraploid tumors the ratio was from 2.3 to 6.79.

AR Expression in Male Breast Tumors

Table 2 lists the histological type, DNA Index (DI), ER expression, AR expression and intensity (as determined by IHC) and percent of AR positive nuclei (by flow cytometry) of the 33 male breast tumors analyzed. 25/33 tumors had predominantly diploid DNA content (DI = 1.0) while 8 of the tumors contained distinct aneuploid sub-populations with near-triploid or hypo-tetraploid DNA content (DI of 1.2-1.6).

Figure 1C shows a plot of DNA content vs. percent of AR positive nuclei seen in sub-populations of the thirty-three breast tumors from male patients. In diploid tumors, the percent of AR positive nuclei varied from 5 to 61 with mean of 31 ± 14. In near-triploid sub-populations, the range was from 23 to 64 with a mean of 44 ± 15. In sub-populations with near-tetraploid DNA content, the range was from 16 to 61 with a mean value of 40 ± 16.

Figure 3 (top row, A-C) shows representative DNA histograms of male breast tumors with diploid and aneuploid subpopulations. The scatter plots (D-F) show DNA content (X-axis) vs. AR expression (Y axis) of nuclei incubated with the anti-AR antibody. Dots above the horizontal line indicate nuclei with positive receptor expression. The overlay histograms in (Fig. 3 G-I) show analysis of the isotype vs. antibody treated samples by the Overton's method.

By IHC, 26 of the 33 male breast tumors were ER positive while only 6 of them had AR positive expression (Table 2). In cytometric analysis using a cut off 20%, 23 tumors were AR positive while at a cutoff of 30%, 10 out of 33 were AR positive. The six AR positive tumors (By IHC) had 23-36% positive AR nuclei in cytometric analysis.

Vitamin D Receptor Expression

Of the seventeen-breast tumor from female patients analyzed for VDR expression, seven had diploid DNA content. Ten of the tumors had besides the diploid cells, distinct aneuploid subpopulations. DNA histograms in Fig 4 show representative tumor populations with diploid DNA content (A), a major triploid sub-population (B) and multiploid (triploid and tetraploid sub-populations, C). Scatter plots (D-F) show DNA content vs. VDR expression in representative tumors shown in histograms A to C. Dots above the horizontal line indicate nuclei with VDR expression. The overlay histograms in Fig 4 (G-O) are from analysis of the isotype vs. antibody treated samples of the total population (first row), the diploid population (middle row) and of the aneuploid population (bottom row) by the Overton's method. In tumors shown in this panel, the diploid tumor had more VDR positive nuclei than the aneuploid tumors and the aneuploid sub-populations in the multiploid tumors did not have significantly greater percentage of VDR positive nuclei than the diploid populations of the same tumor.

Fig 5 shows a plot of DNA content vs. percent of VDR positive nuclei seen in sub-populations of the 17 tumors examined. In diploid tumors the percent of VDR positive nuclei varied from 28 to 86 with mean of 66 ± 21. In near-triploid tumors the range was 68 to 91 with a mean of 70 ± 16. In sub-populations with near tetraploid DNA content, the range was from 36 to 91 with mean of 63 ± 20.

DISCUSSION

Growth of normal and malignant human breast tissue is modulated by a variety of steroid hormones. As several studies have shown that hormone receptor expression of the tumor cells can have both diagnostic and prognostic value, a variety of methods have been used for determination of receptor expression in breast tumors. Ligand binding assays have been replaced by immunohistochemical studies based on the use of antibodies to the nuclear hormone receptors. In contrast to these assays, flow cytometric analysis of the receptor expression in nuclei isolated from archival breast tumors offers the advantage that one can quantitatively determine both the percentage of cells with receptor positive expression as well as determine their antigen density by comparing mean log fluorescence channel ratio of the isotype and the antibody stained samples. The major shortcoming of the flow cytometric method is that unlike immunohistochemistry, one cannot visualize the individual cells and differentiate between receptor expression of the normal and the tumor cells. However, by using DNA content as a second parameter, one can differentiate between normal cells with diploid and tumor cells with aneuploid DNA content. In tumor cells with diploid DNA content (which can not be distinguished from normal cells on the basis of their DNA content), cellular expression of specific markers such as cytokeratins (19) or differences in the nuclear volume (20) may be used to identify tumor cells.

Most of the published work on hormone receptor expression in human breast tumors has focused on ER and PR expression. Several antibodies are commercially available for immunohistochemical evaluation of these receptors. In contrast to the female hormone receptors, there are a limited number of studies on the expression of AR and VDR in human breast tumors. In general, most of the reports indicate that majority of the human breast tumors have highly variable AR expression (21) and often, tumors that are ER or PR negative show AR expression. In a recent publication, Brys et al. (22) reported that 66 and 51 % of breast tumors were AR positive by RT-PCR and western blot, respectively. The number of AR positive cells and the AR mRNA content was higher in tumors than in normal tissues. In this study, 18% of primary breast tumors negative for ER or PR had positive AR expression. Kuenen-Boumeester et al. (4) reported that in grade III tumors loss of ER, PR and AR receptors was accompanied by high proliferation index.

The present study is probably the first one to use flow cytometric methods to show that AR positive nuclei are seen in both ER positive and negative female breast tumors. Furthermore, tumors with high IHC ER staining intensity seem to have higher percentage of AR positive nuclei. In higher grade tumors there is a decrease in percent of AR positive nuclei, although aneuploid subpopulations of multiploid tumors seem to have a higher percentage of AR positive nuclei than the diploid subpopulations.

Human male breast tumors are relatively rare and differ in their biological behavior from tumors of the female breast (23,24). Pacheco et al (25) reported that 75 % of the male breast tumors were AR positive. Pich et al. (26) described that out of the 47 male breast tumors examined, 34 % were AR positive and well-differentiated tumors were more often AR positive than the poorly differentiated tumors. Munoz de Toro et al. (27) have reported that 38.5% of male breast tumors in their study were AR positive and tumors from younger patients showed a significant lack of AR expression. They pointed out that decreased androgen action within the breast tissue may contribute to earlier development of the male breast cancer whereas once the tumor is developed, the presence of AR may contribute to tumor progression as indicated by high proliferative activity in the AR positive cases.

In the present study, we reported AR positive expression in all the thirty-three male breast tumors examined. However, AR expression in male breast tumors was lower than that of the female breast tumors as indicated both by the percent of AR positive nuclei and the antigen density (mean log fluorescence channel ratio).

Our flow cytometric studies confirmed earlier IHC studies suggesting that most of the breast tumor cells have positive VDR expression (5,6). In contrast to AR, VDR expression did not seem to increase in aneuploid female breast tumors and diploid sub-populations had more VDR positive nuclei than the multiploid sub-populations.

In contrast to IHC studies where an observer can use 10% as the cut off for calling a tumor receptor positive or negative, in flow analysis, the range of nuclei with positive expression (after subtraction of the isotype values) can be extensive. In an earlier study (16), we used the AR negative (PC-3), and AR positive cell lines (LnCap) to determine the cut off values, which could be used to identify receptor positive and negative tumors. In AR negative PC-3 cells from log and plateau phase cultures, the percentage of AR positive nuclei were less than 20 % and the ratio of mean log fluorescence channel value (MFC) was less than two. In contrast in the AR positive LnCap cells, the percent of AR positive nuclei increased as cultures reached confluence and reached a maximum of 94% percent positive nuclei and MFC value of 11. Thus one could suggest that these values could be used to differentiate between receptor positive and negative tumors by flow cytometry. Using these criteria one would claim that 23/33 male breast tumors analyzed in the present study (table 2), were AR positive by flow cytometry while IHC identified only 6/33 tumors as AR positive. One of the reasons for discrepancy between the IHC and flow cytometric analysis could be that while the IHC data is based on enumeration of tumor nuclei alone, the flow analysis measures antigen reactivity of both the tumor and the normal nuclei. Thus gated analysis (using DNA content or nuclear volume) and use of specific markers which can differentiate between the normal and the tumor cells could be used to refine the flow cytometric methods for analysis of nuclear hormone receptors in tumors.

As shown in the present study, flow cytometric analysis of nuclear hormone receptor expression can be routinely performed in archival formalin-fixed paraffin-embedded tumors. Data obtained from these studies can be used for correlative studies comparing expression of different cellular markers and possibly for retrospective studies seeking to correlate receptor expression with clinical outcome and response to therapy.

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EXPLANATION TO THE FIGURES:

Figure 1. Plots percent of AR positive nuclei in human female (A, B) and male breast tumors (C). Figure 1A correlates AR expression with tumor grade and low-grade tumors seem to have a higher percent of AR positive nuclei. Figure 1 B shows that diploid sub-populations in the female tumors had lower percent of AR positive nuclei than aneuploid sub-populations. In male tumors AR expression was lower than that of the female tumors.

Figure 2. AR expression in female breast tumors. Figs A-C are DNA histograms of diploid and aneuploid tumors. Figures D-F are scatter plots of AR expression (Y axis) vs. DNA content (X axis). The horizontal line is the gate used to subtract 95 percent of the fluorescent cells from the isotype controls. Histograms G-O are from analysis of the isotype and the antibody treated nuclei by the Overton's method. G-I are of the total population, while J-L and M-O are respectively, of the diploid and the aneuploid sub-populations.

Figure 3. Shows AR expression in male breast tumors. A-C are DNA histograms; D-F are scatter plots of AR vs. DNA content while G-I are Overton's analysis of the AR expression in isotype and antibody treated samples.

Figure 4. VDR expression in female breast tumors. A-C are DNA histograms of diploid and aneuploid tumors. D-F are scatter plots of VDR vs. DNA content. G-I are Overton's analysis of the total population, J-L of diploid sub-populations and M-O of the aneuploid populations.

Figure 5. Correlation of DNA aneuploidy and VDR content of sub-populations in female breast tumors.

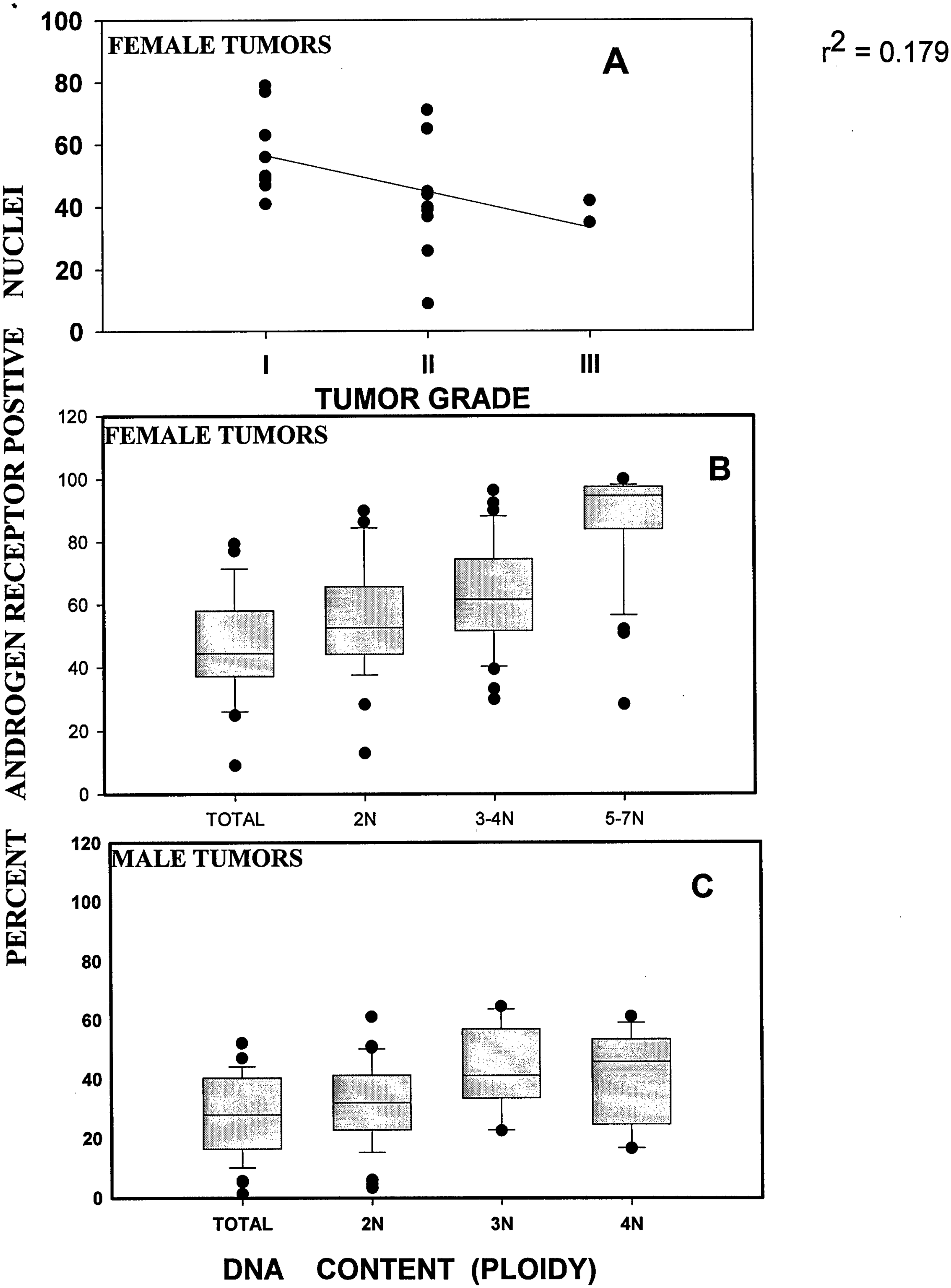
r2 = 0.179
FEMALE TUMORS
A
PERCENT ANDROGEN RECEPTOR POSTIVE NUCLEI
100
80
60
40
20
0
I
II
III
TUMOR GRADE
FEMALE TUMORS
B
120
100
80
60
40
20
0
TOTAL
2N
3-4N
5-7N
MALE TUMORS
C
120
100
80
60
40
20
0
TOTAL
2N
3N
4N
DNA CONTENT (PLOIDY)

fig.1 160902

AR EXPRESSION IN FEMALE BREAST TUMORS

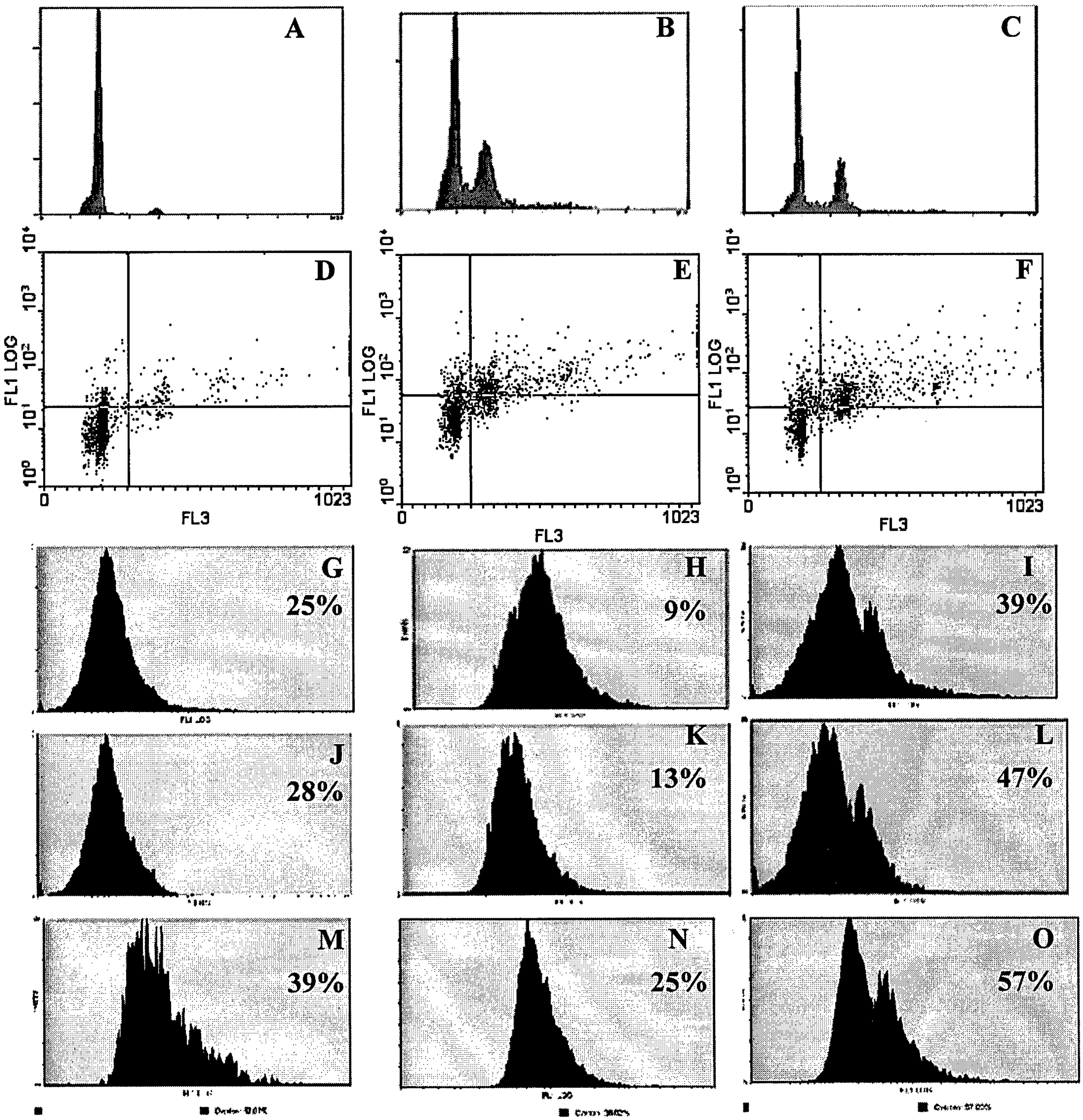

FIGURE 2

Fig 2 AR BT 071002

AR EXPRESSION IN MALE BREAST TUMORS

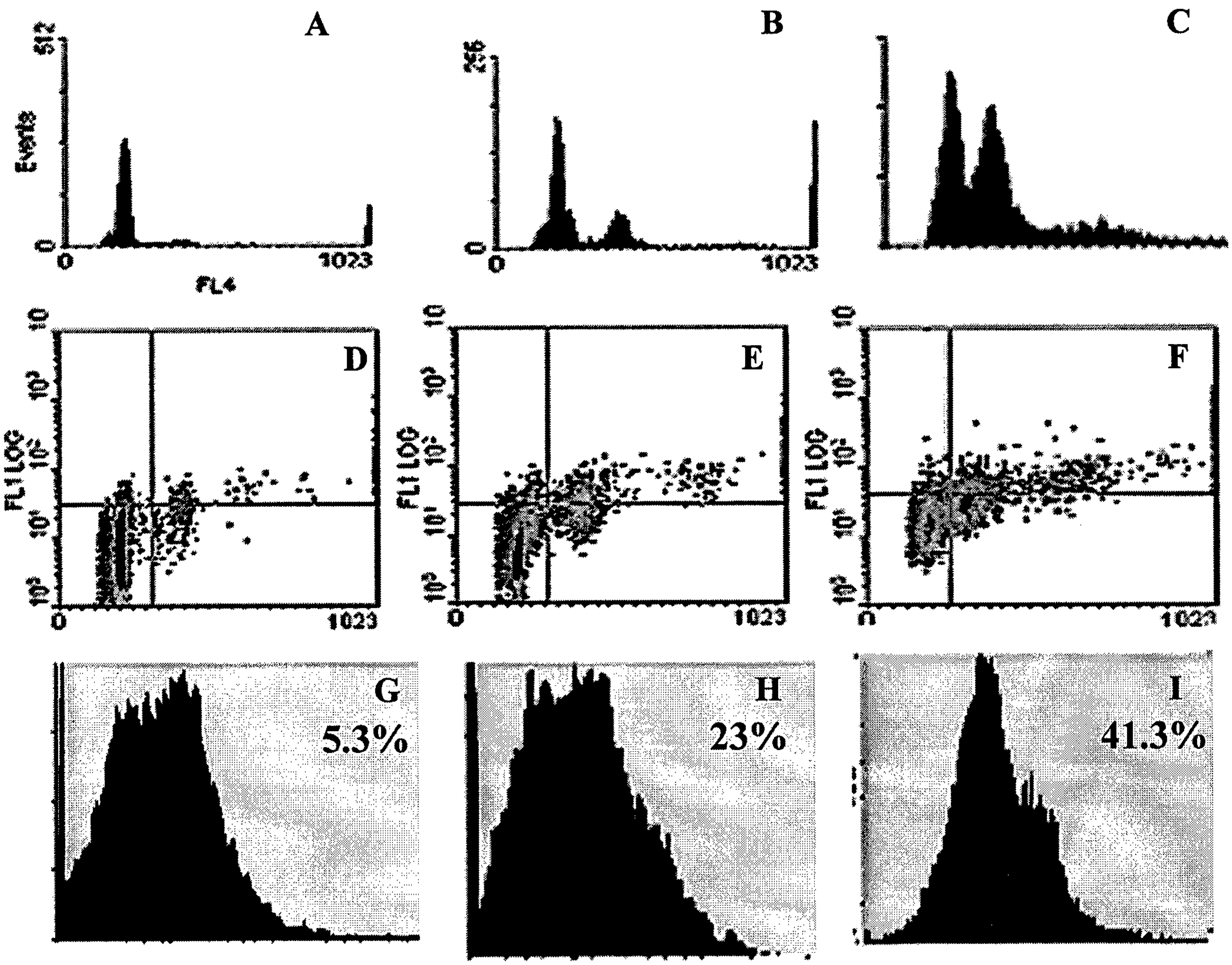

FIGURE 3

FIG 3 AR MALE BT 071002

VDR EXPRESSION IN FEMALE BREAST TUMORS

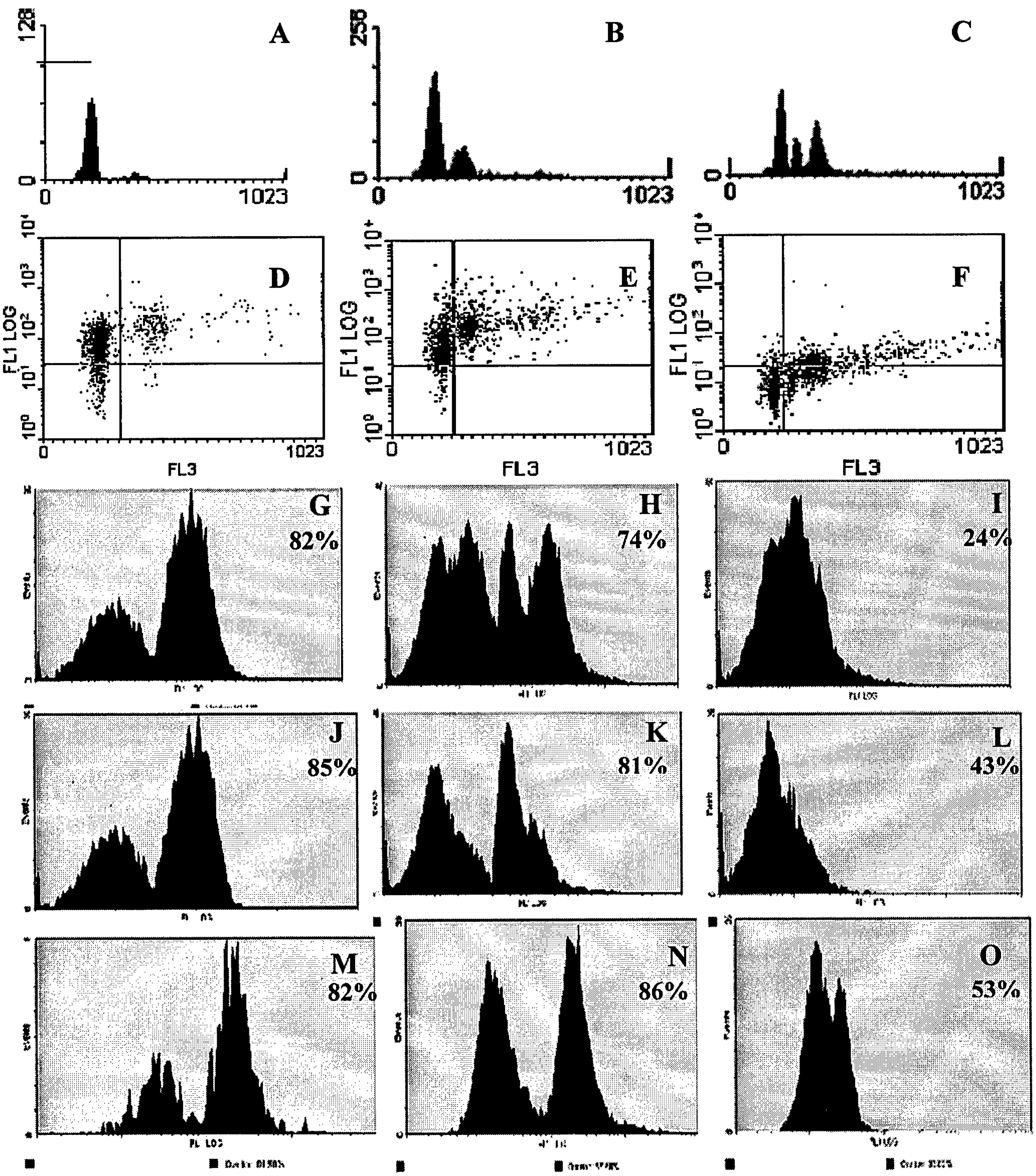

FIGURE 4

FIG 4 VDR BT 071002

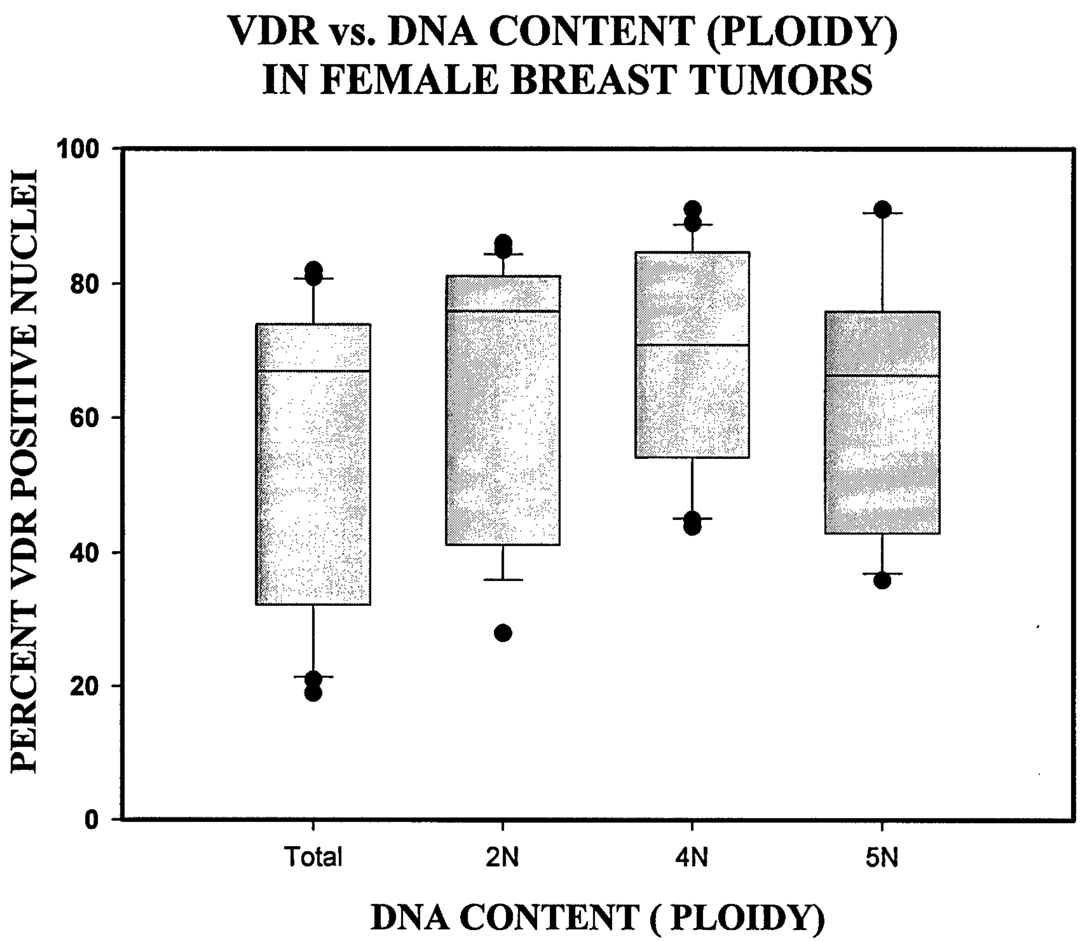

FIGURE 5

Fig.5 vdr vs PLOIDY 071002